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RESEARCH ARTICLE

The phenylthiourea is a competitive inhibitor of the enzymatic oxidation of DOPA by phenoloxidase

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Abstract
Phenoloxidase is a key enzyme of melanization catalyzing the oxidation of phenols. Phenylthiourea (PTU) is the well-known and widely used inhibitor of phenoloxidase. However, the mechanism of its action is not quite clear. In the present work, the effect of PTU on the enzymatic oxidation of 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) by phenoloxidase was studied by spectrophotometric methods. The inhibition constant of PTU was estimated as 0.21 ± 0.09 μM and the competitive type of inhibition was determined for this reaction.

Keywords: Melanization, phenoloxidase, phenylthiourea, enzyme inhibition

Introduction

Melanization is the process of formation of the natural polymer melanin, which is widespread in living organisms. The browning of fruit and root shears and the dark colour of animal skin and hair are mostly caused by melanin formation. This process performs various functions in different organisms, the most important of which is the protective activity. Sometimes, however, the excessive melanin production is related to the development of different diseases such as human melanoma. At present, many researchers are interested in the mechanism of melanin formation, the methods for monitoring the process and its role in organisms[1–4].

The key enzyme of melanization is phenoloxidase ([PO] or tyrosinase; monophenol, ortho-dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) which contains two copper atoms at its active site[5]. This enzyme has three forms: "met" (with Cu(II)-Cu(II) in the active site), "deoxy"(Cu(I)-Cu(I)), and "oxy"(Cu(II)-O_2-Cu(II))[6,7]. Structural models for the active site of these three forms have been proposed[8–14]. PO catalyzes the initial steps of the melanization process, namely, the oxidation by molecular oxygen of monophenols (cresolase activity) and ortho-diphenols (catecholase activity) into the corresponding ortho-quinones. Then these quinones undergo spontaneous intramolecular cyclization to yield indoles which subsequently polymerize into melanin through the cascade of reactions[5].

The initial enzymatic steps of melanogenesis are the key reactions of the whole process. Therefore, they attract great attention in the case of the pigmentation problem caused by melanin formation. At present, many of the melanization inhibitors are known and the mechanisms of their action are quite diverse. A number of inhibitors, such as azides, cyanides, mimosine[8], and dithiocarbamates[15], act directly on the PO inhibiting the whole cascade of the following reactions. Ascorbate[16] and some other compounds interact with the products of the enzymatic reaction to prevent their polymerization. A number of inhibitors, such as cysteine[17], kojic acid[18], captopril ((2S)-N-(3-mercapto-2-methylpropionyl)-L-proline)[19], and thiopronine[20] have a complex effect on the melanization process by interacting with both the active site of PO and the products of the first reactions of melanization (usually with quinone).

Phenylthiourea (PTU) was used as PO inhibitor since the 1940s. It was found that upon different phenols oxidation catalyzed by PO, the oxygen consumption decreased noticeably in the presence of PTU[21]. Later, a

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series of thiourea derivatives were investigated and PTU was found to be the most effective melanization inhibitor[22]. It is commonly accepted that the action of PTU is based on its interaction with copper ions at the active site of PO[21,23–26]. However, some of the authors suppose that PTU is the noncompetitive inhibitor of PO[22,24]. On the other hand, there is an opposite opinion according to which PTU has no effect on the enzyme but interacts only with quinone preventing the subsequent melanin formation[27]. Although PTU is widely used as a specific inhibitor of PO[26,28–31], consistent mechanism of PO inhibition by this compound is not available in the literature. In the present work, we have investigated the inhibition type and estimated the inhibition constant of PTU on the enzymatic oxidation of 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) by PO.

Materials and methods

Reagents

DOPA, PTU, NaCl, diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma (USA).

Mushroom PO was obtained from Worthington (USA). All experiments were carried out in phosphate buffer saline (PBS; 50 mM, pH 7.4, 150 mM NaCl) with 50 μM DTPA. All solutions were prepared with bidistilled deionized water.

High-performance liquid chromatography (HPLC) analysis of the products of DOPA oxidation by PO in the presence of PTU

The 1 mM solutions of DOPA and PTU were used as the standards. A standard solution of 2-carboxy-2,3-dihydroindol-5,6-quinone (DOPA-chrome) was obtained by chemical oxidation of 1 mM DOPA by 2 mM $NaIO_4$[32]. The DOPA-chrome formed was stable for 1 h. The analyzed mixtures contained 1 mM DOPA, 20 U/mL (13 μg/mL) PO, and 1 mM PTU. All the mixtures were prepared in PBS and incubated at room temperature for 50 min. The aliquots of 10 μL from the mixtures were taken for HPLC analysis on the reversed-phase column (Diaspher-110-C16, 150 × 2 mm, 5 μm average particle size, BioChemMak, Russia), using the HPLC system Agilent 1100 (Germany) with the standard software for chromatogram processing (ChemStation A.08.04). The mobile phase contained 3% acetonitrile in 25 mM KH_2PO_4 solution with 1 mM sodium heptylsulfonate as an ion-pair reagent, pH 3.5. The flow rate was 0.3 mL/min. The absorbance values of the effluent were monitored at 280 nm.

Monitoring of DOPA-chrome formation upon DOPA oxidation by PO

The experimental solutions, containing various concentrations of DOPA and 20 U/mL PO, were placed into a quartz cuvette with a 1 cm path length. The rate of DOPA-chrome formation was measured by spectrophotometric technique detecting the absorption at 490 nm ($\varepsilon = 3700\ M^{-1} \times cm^{-1}$) using a UV-2401 (PC) CE spectrophotometer (Shimadzu, Japan). In experiments on inhibition, the PTU of various concentrations was added to the solution of PO before DOPA addition. To check the reversibility of inhibition, PO (200 U/mL) was incubated with PTU (200 μM) for 5 min at 25°C. After that, DOPA solution was added to a final concentration of either 1 mM or 9 mM, simultaneously concentrations of PO and PTU were reduced to 20 U/mL and 20 μM, respectively.

Data analysis

The data were analyzed using the software SigmaPlot 2004 for Windows, version 9.0 (Systant Software, Inc.). When necessary, a statistical analysis was used and the data were expressed as either the means ± SD ($n \geq 3$) or the means ± confidence interval ($n \geq 4$, $p = 0.95$) for K_m and K_i values.

Results and discussion

There are several substances among melanization inhibitors that have no direct effect on the enzyme but prevent melanin formation due to the interaction with the products of this process (e.g. with either quinone or further forming indole compounds). Thus, cysteine associated with quinone to form an achromatic conjugate and the lag period was observed in the kinetic curves of DOPA-chrome formation[17]. Captopril[19] and thiopronine[20] have a similar effect on melanization, forming achromatic product of joining to quinone. In order to check the possibility of the similar conjugate formation upon interaction between DOPA-quinone and PTU, we have analyzed and compared the products of the DOPA enzymatic oxidation without inhibitors and in the presence of PTU using HPLC method (Figure 1). The chromatogram of the reaction mixture of the DOPA oxidation by PO (Figure 1A) demonstrates three peaks: DOPA-chrome

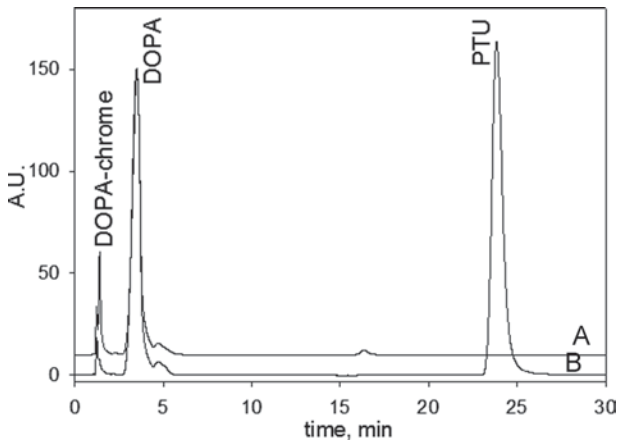


Figure 1. The chromatograms of the reaction mixtures of the 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) oxidation catalyzed by phenoloxidase (PO) in the (A) absence and (B) presence of phenylthiourea (PTU). Incubated mixtures contained 1 mM DOPA, 20 U/mL PO and 1 mM PTU (in the mixture B) in 50 mM phosphate buffered saline (PBS) pH 7.4. Incubation time was 50 min.

(the peak at 1.4 min), DOPA (the peak at 3.5 min), and the third product at 16.5 min, probably DOPA-quinone, because the same peak has been observed in the chromatogram of the DOPA autoxidation mixture (data not shown). Three peaks were also detected by analyzing the reaction mixture of the DOPA oxidation by PO in the presence of PTU (Figure 1B) corresponding to DOPA-chrome, DOPA, and PTU (the peak at 24 min). There were no new peaks corresponding to conjugates of PTU with the enzymatic DOPA oxidation products. The peak detected in the first mixture, presumably of DOPA-quinone, was lacking in this case. Moreover, the intensity of the DOPA-chrome peak was essentially lower in the chromatogram obtained for the mixture with PTU (Figure 1B). Consequently, PTU affects the melanization process by either inhibiting the action of PO directly or reducing DOPA-quinone to DOPA, like ascorbate[16] and kojic acid[18]. In the latter case, kinetic curves of DOPA-chrome formation would have lag period[16,18]. However, the obtained kinetic curves of DOPA oxidation by PO in the presence of PTU have no lag period (Figure 2A). At the same time, the reaction rate was reduced (Figure 2). These findings allow us to make a conclusion that PTU inhibits the melanization process by the interaction with the enzyme.

Usually the efficiency of the inhibition of any enzymatic process is represented as a value of half maximal inhibitory concentration (IC_{50}). The dependence of the reaction rates on inhibitor concentrations was obtained (Figure 2), PTU concentration being varied from 0 to 20 μM in the mixture of DOPA (1 mM) with PO (20 U/mL or 13 μg/ml). According to the data in Figure 2B, the IC_{50} was determined as 0.55 ± 0.07 μM. This finding indicates a quite strong inhibition of PO activity by PTU and is in a good correlation with previous data[5,33]. However, IC_{50} is not a direct indicator of inhibitor-enzyme interaction as it depends on the experimental conditions. The complete value of inhibition is the inhibition constant K_i, which corresponds to the binding affinity of the inhibitor to the enzyme. In order to correctly determine the K_i value, the reversibility of the inhibition process needs to be investigated.

The result of the experiment in which the reversibility of the inhibition was checked is shown in Figure 3. Enzyme was preincubated with PTU to occupy completely the active site of PO by inhibitor and the kinetic curves were obtained for two different concentrations of DOPA. The addition of substrate at 1 mM concentration leads to the negligible reaction rate equal to that of DOPA autoxidation (Figure 3, curve A). While in the presence of the one order higher substrate concentration (9 mM DOPA), the observed kinetics (Figure 3, curve B) had the lag period for about 4 min. The reaction rate increased and reached the constant value equal to the reaction rate under the same conditions without the preincubation of the enzyme with PTU (0.011 μM/s). The activity of PO blocked by interaction with PTU was restored after addition of the excess of DOPA quite quickly, that was demonstrated by the shot lag period (just 4 min). Thus, our data unambiguously indicate that PTU is the reversible inhibitor of PO.

To elucidate the inhibition type and to estimate the inhibition constant K_i, the kinetic study of enzymatic oxidation of DOPA were analyzed. The dependences of the reaction rate on substrate concentration at various inhibitor concentrations are shown in Figure 4A. To determine the type of inhibition, the Lineweaver-Burk, the Hanes-Woolf, and the Eadie-Hofstee linearizations were used. In the case of competitive type of inhibition, the intersection points of the curves must be on the axes on the Lineweaver-Burk and the Eadie-Hofstee plots[34]. All used linearizations presented on Figure 4B, 4C, and 4D indicate that the type of inhibition of PO by PTU is competitive or complex with dominance of competitive type, as the intersection points slightly deviate from ordinate axes on the plots (Figure 4B and 4D) that could indicate the complex type of inhibition. To elucidate the type of inhibition exactly, we used two other linearization

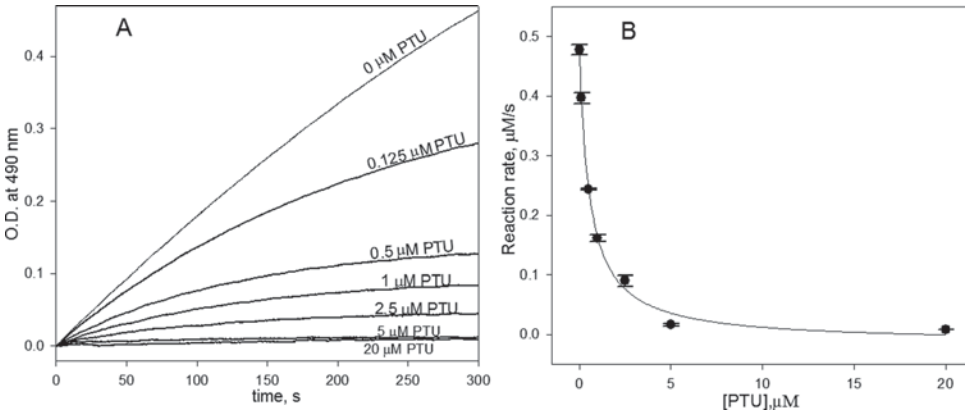


Figure 2. (A) The kinetic curves of the oxidation of 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) catalyzed by phenoloxidase (PO) in the presence of the different concentrations of phenylthiourea (PTU) and (B) the dependence of the reaction rate from the initial linear part of kinetics on PTU concentration. The conditions were as follows: 1 mM DOPA, 20 U/mL PO and various concentration of PTU (0–20 μM) in 50 mM phosphate buffered saline (PBS) pH 7.4.

methods, namely, the Dicson and one-curve method plots (Figure 5A and 5B). The set of curves on these plots completely satisfied the competitive type of inhibition[34]. The inhibition constant for PTU was estimated from each of the used linearization plots. The Michaelis constant for DOPA was estimated from the Lineweaver-Burk, the Hanes-Woolf, the Eadie-Hofstee, and the one-curve method plots. The obtained data compare well with each other and the averaged values were determined as $K_i = 0.21 \pm 0.09$ μM for PTU and $K_M = 0.28 \pm 0.11$ mM for DOPA. To confirm the results, the computer emulation of the reaction rate as a function of substrate concentration by Michaelis–Menten kinetics equation for competitive inhibition was made (Figure 4A). The values of the constants were estimated from the emulation as $K_M = 0.29 \pm 0.06$ mM and $K_i = 0.30 \pm 0.07$ μM. As seen, this result gave the close agreement with the values of constants obtained from linearizations, which supports the competitive type of inhibition of PO by PTU. Moreover, the obtained value of K_M correlates well with K_M for DOPA reported in the literature (0.46 mM ÷ 1.04 mM)[9–11]. Any data concerning K_i value for PTU as an inhibitor of PO were lacking in the literature and were obtained for the first time in the present work.

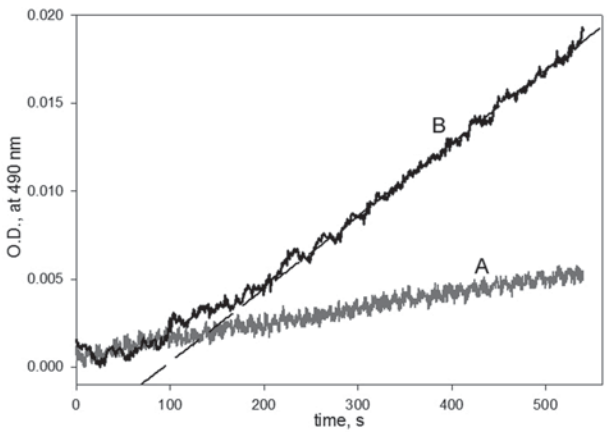


Figure 3. The kinetic curves for checking the reversibility of the inhibition of phenoloxidase (PO) by phenylthiourea (PTU). PO (200 U/mL) was incubated with PTU (200 μM) for 5 min at 25°C. After that, 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) was added to a final concentration of either (A) 1 mM or (B) 9 mM. The final concentrations of PO and PTU were 20 U/mL and 20 μM, respectively. The long dash line is a linear regression fit of reaction rate data.

According to the literature data, the state of the active site of PO passes through three enzymatic forms during the process of the substrate oxidation, namely, Em– met-phenoloxydase, Ed– deoxy-phenoloxydase, and Eo– oxy-phenoloxydase[6,7] (Scheme 1). Em and Eo forms with both copper ions in the oxidation state Cu(II) are the enzymatic forms that bind the substrate, DOPA[10–14]. In case of plant catecholoxidase, it has been shown that it is Cu(II)-CU(II) state of the enzyme that interacts with the PTU[35]. The latter is in agreement with our finding that PTU interacts with Cu(II) (CuSO4) producing insoluble compound (data not shown). Therefore, it allows us to hypothesize that competitive mechanism of the inhibition of DOPA oxidation by PO is a consequence

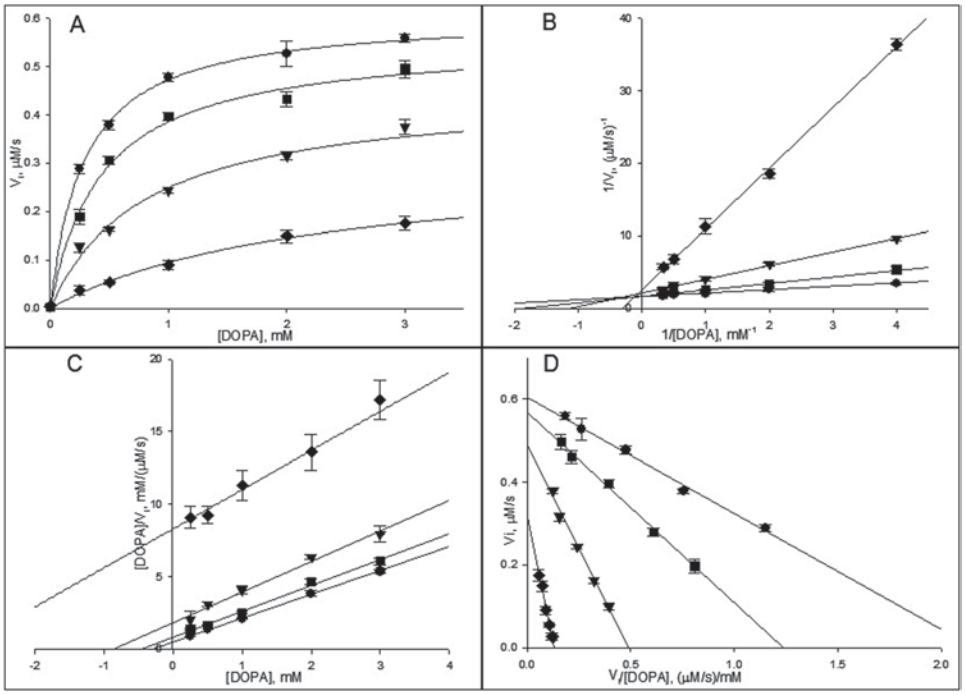


Figure 4. (A) The dependences of the reaction rates of 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) oxidation catalyzed by phenoloxidase (PO; 20 U/mL) on substrate concentration in the absence (•) and in the presence of phenylthiourea (PTU): 0.125 μM (■), 0.5 μM (▼), 2.5 μM (◆). —, nonlinear regression fit of data to Michaelis–Menten equation for competitive inhibition. The linearizations of these dose-dependent curves: (B) the Lineweaver-Burk plot, (C) the Hanes-Woolf plot and (D) the Eadie-Hofstee plot. The symbol meanings are the same that in (A).

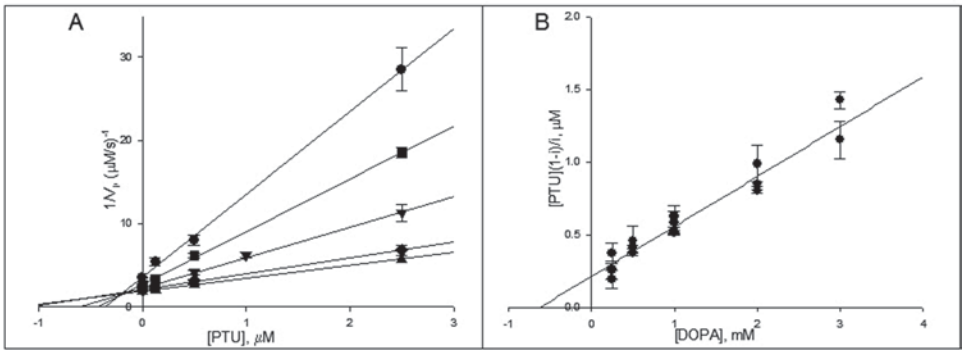
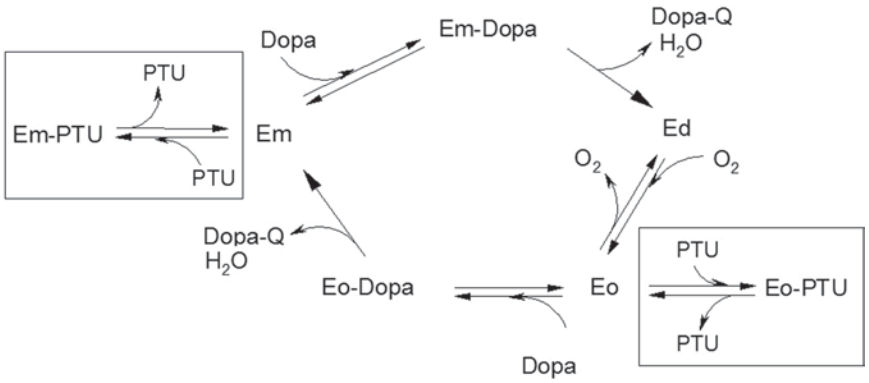


Figure 5. The Dicson (A) and the one-curve method (B) linearizations of the data presented in Figure 4 A. The symbols meanings are (•) 0.25 mM, (■) 0.5 mM, (▼) 1 mM, (◆) 2 mM and (▲) 3 mM of 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA).



Scheme 1. Simplified kinetic scheme which describes the effect of phenylthiourea (PTU) on the 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) oxidation catalyzed by phenoloxidase (PO). The processes in the presence of PTU are marked by frames. The enzymatic forms of PO are as follows: Em– met-phenoloxydase (with Cu(II)-Cu(II) in the active site), Ed– deoxy-phenoloxydase (with Cu(I)-Cu(I) in the active site), Eo– oxy-phenoloxydase (with Cu(II)-O_2-Cu(II) in the active site).

of competitive binding of DOPA and PTU to the "met" and "oxy" forms of the enzyme as illustrated in the Scheme 1.

Our results revealed that K_i value for PTU is an order less than that for such known inhibitors of PO as dithiocarbamates (K_i ~ 10–20 μM)[15]. This indicates that PTU has an advantage with respect to dithiocarbamates, mimosine, and cyanide [8] in its ability to inhibit PO.

In summary, the properties of PTU as inhibitor of enzymatic oxidation of DOPA by PO were studied. The results obtained show that the influence of PTU on this process is caused by the interaction of the inhibitor with PO rather than with intermediate products of DOPA oxidation. The inhibition constant of PTU in this reaction was determined ($K_i = 0.21 \pm 0.09$ μM) and the type of inhibition was clearly shown to be competitive. Our data indicate that PTU is a very strong inhibitor of PO.

Declaration of interest

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